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Computerized Nuclear Morphometry of Hepatocellular Carcinoma and Its Relation to Proliferative Activity

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Background and Objectives: Nuclear profiles have been reported as useful prognostic predictor in various cancers. Data from computerized morphometries are objective and can be quickly derived using conventional microscopic analysis. However, it remains to be shown what types of pathological and biological factors influence the nuclear features. The aim of this study was to evaluate the correlation between the morphological nuclear features and clinicopathological parameters in patients with hepatocellular carcinoma (HCC).

Methods: Morphometric nuclear features (nuclear area, perimeter, and shape) were analyzed in 76 patients with hepatocellular carcinoma who underwent hepatectomy at our hospital. In each case, 300 cancer nuclei were analyzed randomly on routine hematoxylin&eosin-stained slides through the use of a computer-assisted image analysis system that allowed us to trace the nuclear profiles (magnification ×400) on a computer monitor. The morphometric data were compared with patient survival, clinicopathologic status, and the proliferative activity of cancer cells.

Results: The mean nuclear area of poorly differentiated carcinoma was significantly larger than that of moderately and well differentiated carcinoma ($P = 0.0003$). Significant correlation was detected between the nuclear area of cancer cells and proliferative activity associated with proliferating cell nuclear antigen labeling index (PCNA LI) of cancer cells ($r = 0.372$, $P = 0.0008$). Moreover, blood vessel invasion of cancer cells or intrahepatic metastasis were more frequently detected in patients with large nuclear areas. Even though the nuclear area was not an independent prognostic factor in the multivariate analysis, the 5-year survival rate of the 35 patients who had tumors with large nuclear areas (>50 μm^2, 25.9%) was significantly lower than that of the 36 patients who had tumors with small nuclear areas ($\leqslant 50$ μm^2, 63.3%, $P = 0.001$).

Conclusions: The nuclear area of HCC correlates with cell differentiation and cell proliferative activity. Moreover, HCC with a large nuclear area has high potential for blood vessel invasion and intrahepatic metastasis. Thus, nuclear morphometry can be used as an useful morphological predictor for malignant potential in patients with HCC.

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KEY WORDS: **nuclear area; hepatocellular carcinoma; blood vessel invasion; intrahepatic metastasis**

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INTRODUCTION

Several parameters, including DNA ploidy [1], detection of the expression of cancer-associated antigen [2], and loss of the tumor suppressor genes in cancer cells [3,4] have been offered as prognostic indicators for patients with hepatocellular carcinoma (HCC). However, determination of these parameters involves complex and difficult techniques. The existence of heterogeneous DNA ploidy or heterogeneous expression of oncogene products in tumors should be considered. Moreover, results from these methods are often inconclusive.

Nuclear profiles, such as the size of the nuclear area or the shape of the nucleus, have recently been suggested as useful elements of prognosis in various cancers [5–8]. Interactive computerized morphometry is a quantitative technique that measures the dimensions of nuclear size and the shape of cancer cells. The resulting data are objective, and the technique can be quickly performed using conventional microscopic analysis. Moreover, intratumoral differences in nuclear size have not yet been reported [9]. Thus, in this study, we evaluated the value of nuclear morphometric analysis as a prognostic indicator in HCC. The morphometric data from 76 patients with HCC who were treated with resection at our hospital were compared with respect to patient clinicopathologic status, survival, and the proliferative activity of cancer cells.

PATIENTS AND METHODS

Patients

Between 1987 and 1994, 76 patients underwent hepatectomy for HCC at our hospital. None had received preoperative chemotherapy. All patients were followed up at our hospital. Forty-two patients had died by the end of 1997. Causes of death were determined from clinical findings and, in some cases, an autopsy was performed. Thirty-one of 42 patients died from recurrence of HCC, six patients died from causes other than HCC, and five patients died of postoperative complications within 30 days after surgery.

Histopathologic Examination

The resected specimens were fixed in 10% buffered formalin. All specimens were cut into slices after formalin fixation. The slices were embedded in paraffin, and serial sections (4 μm thick) were prepared and stained with hematoxylin&eosin (H&E) for histopathological diagnosis. Pathological evaluations were assessed by reference to the criteria of the Japanese Research Society for hepatocellular carcinoma [10].

Computerized Nuclear Morphometry

The specimens were taken from the HCC and the noncancerous liver tissue adjacent to the carcinoma in each patient. The slides stained with H&E were analyzed morphometrically by an operator who had no knowledge of the patients. Pathological archival H&E-stained sections of tumors and noncancerous liver tissues adjacent to tumors were viewed under a high-power field (×400, VANOX-S, Olympus, Tokyo). Each image was visualized on a computer display (Macintosh 7500/100, Apple Computer, Cupertino, CA) using a color video camera module (XC-003, SONY, Tokyo) and color image freezer (AE-6905C, ATTO, Tokyo). For each specimen, 30 images of cell fields were captured by the operator, who moved the microscopic field randomly across the specimen. For each slide, 300 nuclei with complete and clearly identifiable outlines, in nonoverlapping and nonfragmented cells, were measured. The outline of each nucleus on the display was traced using a computer mouse. The perimeters and areas of 300 cell nuclei were calculated using PI-2 software (ATTO). The shape of each nucleus was automatically determined by a mathematical transformation:

$$(4 \times \pi \times \text{area}/\text{perimeter}^2).$$

The calculated shape was dimensionless, its value equaling 1.0 for a perfect circle and less than 1.0 for an ellipse [6]. The means of the areas, perimeters, and shapes of 300 cancer nuclei and 300 noncancerous liver cell nuclei from each patient were registered and compared.

Immunohistochemical Staining

Serial sections of 4-μm thickness, taken from the same paraffin blocks used for morphometric analysis, were immunostained using monoclonal antibody raised against proliferating cell nuclear antigen (PCNA) (PC-10; diluted 1:20) (DAKO, Carpinteria, CA). The methods for immunostaining were described previously [11,12]. Immunostained sections of 76 tumors and 76 noncancerous liver tissues from 76 patients with HCC were analyzed under a light microscope by two observers who did not know any details of any case. All labeled nuclei were regarded as positive. To determine the average frequencies of immunolabeling with PCNA in nuclei, 20 microscopic fields were monitored randomly in each sample and 1,000–2,000 cancer cells were examined. The average frequency of immunolabeling of nuclei with PC-10 in each case was expressed as the PCNA labeling index (LI), the percentage of immunostained cancer cells.

Statistical Analysis

To compare frequencies, Fisher's exact probability test and the chi-square test were used. The differences in the numerical data between the two groups were evaluated by Student's *t*-test or the Mann-Whitney U-test. The relationships between various parameters were evaluated statistically, using the Spearman rank correlation test.

Five patients who died from postoperative complications were excluded from the univariate and multivariate survival analysis. Survival rates were calculated for the remaining 71 patients by the Kaplan–Meier method. Corrected survival rates were used; that is, only deaths caused by HCC were taken as outcome events; all other deaths were considered censored events. The generalized Wilcoxon test was used for comparisons of the two survival curves. Multivariate survival analysis was performed using Cox's proportional-hazard model. Thirteen variables were entered into the model: age, gender, histologic type of tumor (well-differentiated, moderately differentiated, or poorly differentiated), maximum diameter of the tumor, serosal invasion (absent or present), blood vessel invasion (absent or present), intrahepatic metastasis (absent or present), surgical-free margin (absent or present), stage (I, II, or III, IV), surgical curability (curative resection or noncurative resection), PCNA LI, nuclear area, and nuclear shape, *P*-values of < 0.05 were considered statistically significant.

RESULTS

The clinicopathological characteristics of 76 patients with HCC are presented in Table I. The mean PCNA LI of 76 tumors (23.5 ± 19.1%) was significantly higher than that of 76 noncancerous liver tissues adjacent to tumors (3.4 ± 2.6%, $P < 0.0001$). The mean size of nuclear areas of 76 tumors (53.9 ± 21.7 μm^2) was significantly larger than that of 76 noncancerous liver tissues adjacent to tumors (38.5 ± 5.4 μm^2, $P < 0.0001$). A significant correlation was observed between the nuclear area and the PCNA LI in 76 tumors ($r = 0.372$, $P = 0.0008$, Fig. 1). However, there was no significant correlation between the PCNA LI and the nuclear area in 76 noncancerous liver tissues ($r = 0.178$, $P = 0.1247$). Significant correlations between the histopathologic types of HCC and the PCNA LI or the size of nuclear areas of tumor cells were detected (Table II).

Patients were divided into two groups, according to the size of the nuclear areas of their tumors. Thirty-nine patients with nuclear areas of $\leqslant 50$ μm^2 were classified as a small nuclear group, and 37 patients with nuclear areas of more than 50 μm^2 were classified as a large nuclear group. The clinicopathological characteristics of these two groups were compared (Table III). Blood vessel invasion of cancer cells and intrahepatic metastasis were more frequently detected in the large nuclear group (51.3%, 43.2%) than in the small nuclear group (20.5%, 12.8%, $P = 0.01$, $P = 0.007$). The mean nuclear area of 27 tumors with blood vessel invasion (65.5 ± 28.4 μm^2) was significantly larger than that of 49 tumors without blood vessel invasion (47.6 ± 13.6 μm^2, $P = 0.0011$). Moreover, the mean nuclear area of 21 tumors with intrahepatic metastasis (70.1 ± 29.4 μm^2) was significantly larger than that of 55 tumors without intrahepatic metastasis (47.7 ± 14 μm^2, $P = 0.0003$). The PCNA LI of 37 tumors with large nuclei was significantly higher than that of 39 tumors with small nuclei ($P = 0.00029$). However, no significant correlation was detected between the size of nuclear area and the nuclear shape in 76 tumors ($r = -0.134$, $P = 0.2499$). Also, no significant correlations were observed between the tumor size and the nuclear area ($r = 0.095$, $P = 0.4168$) and between the tumor size and the PCNA LI ($r = 0.085$, $P = 0.4659$) in 76 tumors.

The 5-year survival rate among the 71 surviving patients was 45%. The 5-year survival rate of the 36 patients in the small nuclear group (63.3%) was significantly better than that of the 35 patients in the large nuclear group (25.9%, $P = 0.001$) (Fig. 2). Maximum tumor size and blood vessel invasion were recognized as independent prognostic factors by multivariate analysis. However, the size of nuclear area was not detected as an independent prognostic factor by multivariate analysis ($P = 0.632$) (Table IV).

TABLE I. Background of 76 Patients With Hepatocellular Carcinoma*

Variables	
Age (yr)	Mean ±SD: 57.9 ± 10.3, range: 17–80, median: 61
Gender (male/female)	60/16
Histologic type of tumor	
Well differentiated	8
Moderately differentiated	52
Poorly differentiated	16
Maximum diameter of tumor (mm)	Mean ±SD: 38 ± 24, range: 7–110, median: 30
Serosal infiltration (absent/present)	65/11
Blood vessel invasion (absent/present)	49/27
Intrahepatic metastasis (absent/present)	55/21
Surgical-free margin (absent/present)	34/42
Stage	
I	13
II	36
III	10
IV-A	17
Surgical curability	
Curative operation	36
Noncurative operation	40
PCNA LI (%)	Mean ±SD: 23.5 ± 19.1, range: 0.5–75.5, median: 18
Nuclear area (μm^2)	Mean ±SD: 53.9 ± 21.7, range: 20–168.9, median: 50
Nuclear perimeter (μm)	Mean ±SD: 24.1 ± 3.7, range: 17.3–48, median: 25.7
Nuclear shape	Mean ±SD: 0.96 ± 0.03, range: 0.84–1.02, median: 0.97

*Nuclear shape was defined by the formula: $4 \times \pi \times$ area/perimeter^2. LI, labeling index; PCNA, proliferating cell nuclear antigen; SD, standard deviation.

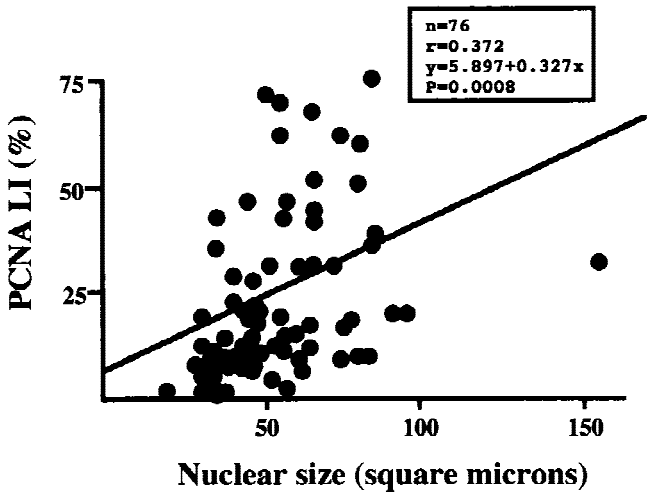

Fig. 1. Significant correlation was detected between the proliferating cell nuclear antigen labeling index (PCNA LI) and the size of nuclear area of 76 tumors with hepatocellular carcinoma.

TABLE II. Comparison of PCNA LI and Nuclear Area in Three Different Histopathologic Types of Hepatocellular Carcinoma*

	No. of cases	PCNA LI (mean ± SD, %)	Nuclear area (mean ± SD/μm^2)
Well differentiated	8	9.2 ± 2.9 (range: 6–15)	41 ± 12.9 (range: 28.1–60.9)
Moderately differentiated	52	22.9 ± 18.9 (range: 0.5–72)	50.2 ± 16.2 (range: 20–95.5)
Poorly differentiated	16	32.9 ± 20 (range: 2–75.5)	72.6 ± 29.6 (range: 38.2–168.9)

*There was a significant difference in the PCNA LI among three different histopathologic types of hepatocellular carcinoma ($P = 0.0025$). There was a significant difference in the nuclear area among three different histopathologic types of hepatocellular carcinoma ($P = 0.0003$). The statistical analyses were performed by the Kruskal-Wallis test.
LI, labeling index; PCNA, proliferating cell nuclear antigen.

DISCUSSION

Computerized image analysis allows accurate and objective evaluation of nuclear morphology, and has been used to show that increases in nuclear size and in irregularity of nuclear shape are more frequently detected in carcinomas than in nonmalignant borderline tumors [8,13]. Increasing abnormalities of nuclear morphometric features in conjunction with tumor progression have been reported in thyroid tumor [14], colorectal cancer [6], renal cell carcinoma [7,15] and breast cancer [5,9,16,17]. Moreover, nuclear size has been reported as an important prognostic factor for patients with intraoral squamous cell carcinoma [18], breast cancer [5], and renal cell carcinoma [7,15], whereas nuclear shape has been reported to be an important prognostic factor for patients with colorectal carcinoma [6]. Thus, abnormal nuclear size and shape appears to be a generalized condition among neoplasia. However, in HCC, the correlation between the

TABLE III. Comparison of Clinicopathological and Biological Characteristics of Tumors According to the Size of Nuclear Area of Cancer Cells*

Variables	Small nuclear group[a] (n = 39)	Large nuclear group[b] (n = 37)	*P*
Age (yr, mean ± SD)	61.2 ± 8.8	58.2 ± 11.6	0.216
Gender (male/female)	30/9	30/7	0.871
Histologic type			
Well differentiated	6	2	
Moderately differentiated	31	21	0.002
Poorly differentiated	2	14	
Maximum diameter of tumor (cm, mean ± SD)	3.7 ± 2.3	4 ± 2.5	0.578
Serosal infiltration (absent/present)	35/4	30/7	0.455
Blood vessel invasion (absent/present)	31/8	18/19	0.01
Intrahepatic metastasis (absent/present)	34/5	21/16	0.007
Surgical-free margin (absent/present)	20/19	14/23	0.343
Stage			
I	5	8	
II	25	11	
III	3	7	0.027
IV-A	6	11	
Surgical curability			
Curative operation	22	14	0.164
Noncurative operation	17	23	
PCNA LI (%, mean ± SD)	15.9 ± 14.2	31.5 ± 20.5	0.00029
Nuclear perimeter (μm, mean ± SD)	22.6 ± 2.1	30.1 ± 4.1	<0.0001
Nuclear shape (mean ± SD)	0.97 ± 0.03	0.96 ± 0.04	0.23

*LI, labeling index; PCNA, proliferating cell nuclear antigen.
[a]Nuclear area $\leq 50\ \mu m^2$.
[b]Nuclear area $>50\ \mu m^2$.

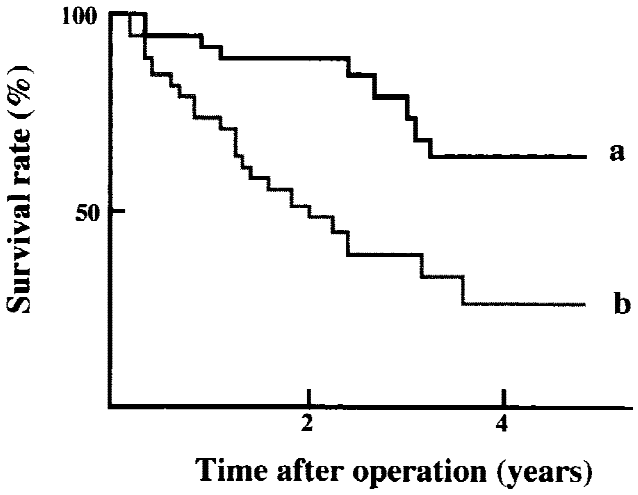

Fig. 2. The 5-year survival rate of the 36 patients in the small nuclear group (nuclear area $\leq 50\ \mu m^2$) was 63.3% (curve a). The 5-year survival rate of the 35 patients in the large nuclear group (nuclear area $>50\ \mu m^2$) was 25.9% (curve b). Significant difference was detected between the two survival curves by the generalized Wilcoxon test ($P = 0.001$).

TABLE IV. Results of Multivariate Survival Analysis of Possible Prognostic Factors for 71 Surviving Patients by Cox's Proportional-Hazard Model*

Variables	Coefficient	Standard error	*P*	Relative risk	Relative risk–confidence interval
Age	0.001	0.022	0.97	1.001	0.958–1.046
Gender					
Male (n = 56)	−1.14	0.871	0.191	0.32	0.058–1.766
Female (n = 15)					
Histologic type[a]					
Well, moderate (n = 55) poor (n = 16)	−0.529	0.569	0.353	0.589	0.193–1.799
Maximum diameter of tumor	0.018	0.009	0.047	1.019	1–1.037
Serosal infiltration					
Absent (n = 61)	−0.539	0.603	0.371	0.583	0.179–1.9
Present (n = 10)					
Blood vessel invasion					
Absent (n = 46)	−1.865	0.576	0.001	0.155	0.05–0.48
Present (n = 25)					
Intrahepatic metastasis	0.029	0.604	0.962	1.029	0.315–3.364
Absent (n = 50)					
Present (n = 21)					
Surgical-free margin					
Absent (n = 32)	−0.501	0.518	0.334	0.606	0.22–1.673
Present (n = 39)					
Stage					
I, II (n = 45)	1.21	0.622	0.052	3.352	0.991–11.343
III, IV (n = 26)					
Surgical curability					
Curative (n = 34)	−0.831	0.674	0.218	0.436	0.116–1.634
Noncurative (n = 37)					
PCNA LI	0.006	0.012	0.591	1.006	0.983–1.03
Nuclear area	0.005	0.01	0.632	1.005	0.985–1.026
Nuclear shape	−7.011	5.827	0.229	0.001	0–82.315

*LI, labeling index; PCNA, proliferating cell nuclear antigen.
[a]Well, moderately, and poorly differentiated.

clinicopathological and biological characteristics and the nuclear morphology of cancer cells has not been clear.

In this study, we analyzed the relationship between the nuclear parameters and the clinicopathological characteristics in 76 patients with HCC. Among the nuclear parameters studied, nuclear area was more informative and more reproducible than nuclear shape. The size of the nuclear area increased from noncancerous cirrhotic liver cells to well-differentiated carcinoma, increasing from well-differentiated or moderately-differentiated to poorly-differentiated carcinoma. Thus, the size of nuclear area of HCC was significantly correlated with cell differentiation. Moreover, a strong correlation was detected between the nuclear size and blood vessel invasion of cancer cells. These results indicate that cancer cells with a large nuclear area should have a high potential to invade the microvessels in the liver; as a result, intrahepatic metastasis was more frequently detected in patients who had tumors of large nuclear area.

It is now widely accepted that genetic alterations play an important role in the development of HCC. However, the specific types of biological behavior that influence the nuclear morphology of cancer cells are unclear. We investigated the relationship betwee nuclear morphology and the percentage of positive cancer cells with PCNA as an indicator of proliferative activity of cancer cells in HCC. PCNA is a highly conserved 36-kD acidic nuclear protein and its expression is necessary for DNA synthesis [19]. PCNA has been found to be a useful marker in analysis of cell kinetics because its expression and distribution correlate with the rate of cell proliferation and DNA synthesis. In this study, we detected a significant correlation between the nuclear areas of cancer cells and PCNA LI of tumors. Mulder et al. [13] reported that nuclear area showed a trend toward correlation with allelic loss on 5q, 18q, and 17p. These findings strongly suggest that nuclear morphology may be influenced by accumulated alterations in cancer-associated genes. This hypothesis remains to be clarified.

Nuclear morphometry is an objective and reproducible procedure that is relatively simple to perform. Our study showed that the nuclear area is closely correlated with the malignant potential of HCC. Therefore, nuclear morphometry should be introduced as a new and useful morphological prognostic marker for cancer patients.

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